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Synergistic antinociceptive actions and tolerance development produced by morphine–fentanyl coadministration: Correlation with µ-opioid receptor internalization

Arturo Silva-Moreno a, Claudia Gonzalez-Espinosa a, Martha León-Olea b, Silvia L. Cruz a,b,⁎

a Departamento de Farmacobiología, Cinvestav, Sede Sur. Calzada de los Tenores # 235, Col. Granjas Coapa, México D.F., 14330, México

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A B S T R A C T

It has been described that coadministration of opioids with low doses of other analgesics can reduce adverse effects and increase antinociception, but combinations of two µ-opioid receptor agonists have been poorly explored. The objective of this work was threefold: 1) to evaluate the antinociceptive combination of i.c.v. morphine and fentanyl at different doses; 2) to compare the antinociception produced by acute or repeated administration of an effective morphine dose (1 µg) alone, or combined with a low fentanyl dose (1 ng); and 3) to correlate these effects with µ-opioid receptor internalization in periaqueductal gray matter and locus coeruleus. Antinociception was evaluated by the tail-flick test and receptor internalization was analyzed by confocal microscopy in Wistar rats. Drug interactions were examined by administering combinations of opioids in 1:3, 1:1 and 3:1 ratios of their respective ED50 fractions. For tolerance and internalization studies, animals were i.c.v. injected only once (acute treatment) or twice a day until five administrations were completed. Our results show that morphine and fentanyl have synergistic effects. The combination of 1 ng fentanyl with 1 µg morphine increases the magnitude and duration of antinociception not only after a single injection, but also after five administrations when tolerance develops to morphine alone. Increased and long-lasting antinociception correlates positively with increased β-arrestin 2 activity and µ-opioid receptor internalization in periaqueductal gray matter and locus coeruleus. These results suggest that combined administration of morphine and fentanyl increases long-lasting antinociception and β-arrestin 2 signaling contributes to the combination effects.

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1. Introduction

Morphine and fentanyl are effective µ-opioid receptor analgesics, but their repeated administration can produce tolerance and physical dependence (Trescot et al., 2008; Waldhoer et al., 2004; Walwyn et al., 2010). A strategy to attenuate opioid–induced adverse effects is to combine them with low doses of non-steroidal anti-inflammatory drugs (Goldstein, 2002; Silva-Moreno et al., 2009) or other opioids (Smith, 2008).

Controlled studies of combined opioids are scarce and their results, controversial (Mercadante et al., 2004, Dottrens et al. (1992) reported that the analgesic effects of a morphine–sufentanil combination after surgery lasted longer than those of morphine alone. In contrast, Friedman et al. (2008) did not find benefits from a morphine–fentanyl combination in patients recovering from surgery. In preclinical studies, synergistic effects have been observed between l-methadone and other µ-opioid receptor agonists, including morphine (Bolan et al., 2002).

Opioid analgesics differ in efficacy (Sirohi et al., 2008), affinity for different µ-opioid receptor isoforms (Pasternak, 2004; Xu et al., 2011), activation of transcriptional factors (Zheng et al., 2010a), and β-arrestin-dependent receptor internalization (von Zastrow, 2010). Arrestin-dependent signals can be pharmacologically dissociated from those dependent on G-protein activation by using “biased” ligands that induce differential coupling of G-protein coupled receptors favoring one pathway or the other. High internalization capacity has been associated with preferential β-arrestin signaling, whereas low endocytosis is considered an indicator of preferential G-protein dependent activation (Luttrell and Gesty-Palmer, 2010). Fentanyl, a β-arrestin-biased ligand (Zheng et al., 2008, 2010b), readily induces receptor endocytosis and recycling (Bohn et al., 2004), whereas morphine, a G protein-biased ligand, produces lower internalization (Beaulieu, 2005; Rajagopal et al., 2010).

Acute coadministration of morphine with low doses of 2-Ala-4-mephe-5-gly-enkephalin (DAMGO) or methadone, two µ agonists with high internalization capacity, enhances antinociception and
To reduce the stress associated with experiments, rats were handled surgery on Friday and were allowed to recover during the weekend. fi

-1.4, and experimental pain in animals (Zimmermann, 1983).

2.2. Drugs

Morphine sulfate and fentanyl citrate were obtained via the Mexican Health Secretariat. Ketamine hydrochloride was bought from Probiomed (Mexico City, Mexico) and xylazine hydrochloride, from Pisa (Tula Hidalgo, Mexico). All analgesic drugs were dissolved in sterile saline solution and administered i.c.v., in a final volume of 5 μl during 1 min, using an infusion pump (KD Scientific, USA). This volume was kept constant whether opioid drugs were administered individually or in combination.

2.3. Surgical procedure

Rats were anesthetized with a mixture of ketamine/xylazine (45/12 mg/kg, i.p.) and mounted on a stereotaxic apparatus to place a permanent guide cannula (10 mm long) in the right lateral cerebral ventricle using the following coordinates from bregma: — 1.5, midline: 1.4, and — 4 from dura (Paxinos and Watson, 1986). The cannula was fixed with two screws and dental cement. Animals underwent surgery on Friday and were allowed to recover during the weekend. To reduce the stress associated with experiments, rats were handled for 15 min twice a day, during 2 additional days in the experimental environment, with the injection cannula inserted into the guide cannula. Nociception studies were performed at least five days after surgery. Once experiments were completed, animals were perfused with paraformaldehyde and brain coronal sections were observed in the microscope to corroborate the injection site.

2.4. Antinociception assessment

We used a tail-flick apparatus with a radiant heat source connected to an automatic timer (Ugo Basile, Italy) and determined the increase in tail withdrawal latency for nociception assessment (Nance and Sawynok, 1987). The stimulus intensity was adjusted at the beginning of the study to get a baseline tail-flick latency of 6.0 ± 0.5 s. Each animal was tested three times before the first drug injection to obtain a mean baseline latency value. Rats that showed no flicking within 5.5 to 6.5 s (5 to 10% of the total) were discarded. We established a cutoff time of 15 s to avoid tail skin tissue damage. Tail withdrawal latency was recorded every 15 min during the first hour after drug administration, and every 30 min in the second hour.

2.5. Protocol

Ten independent groups of rats (n = 6, each) were used to determine the dose–response relationships for acute i.c.v. administration of fentanyl (0.001, 0.01, 0.031, 0.1 or 0.2 μg) or morphine (0.31, 0.56, 1.0, 3.1 or 5.6 μg). Each animal was tested only once. Based on these curves, we selected the doses to be used in combination for synergistic analysis, tolerance development and μ-opioid receptor internalization.

For chronic treatment, four independent groups of animals were used (n = 9, each). Rats received five injections (at 8:00 and 20:00 h until treatment was completed) of saline solution, 1 μg morphine, 1 ng fentanyl or the combination of these opioids at the same doses. In all cases, the final injection volume was 5 μl. Nociception was evaluated only after the morning dose to minimize exposure to noxious stimuli. After completing treatment, 6 animals from each group were used to evaluate withdrawal signs after a challenge naloxone dose (31 μg, i.c.v.) administered 30 min after the last morphine injection, and precipitated abstinence signs were monitored for 30 min. The remaining animals were used for immunohistochemistry analysis.

2.6. Immunohistochemistry

To evaluate μ-opioid receptor presence, β-arrestin 2 activation and changes in μ-opioid receptor cell localization, independent groups of rats (n = 3, per group) were deeply anesthetized with sodium pentobarbital 15 min after one or five administrations of each treatment and then perfused intracardially with 200 ml of 0.1 M saline phosphate buffer (pH 7.2–7.4), followed by 200 ml cold 4% paraformaldehyde in the phosphate buffer. The brain was dissected, removed, and post-fixed during 4 h in the paraformaldehyde solution at 4 °C. Finally, the brain was cryoprotected at least 48 h in 30% sucrose buffer solution.

Coronal sections (30 μm thick) of periaqueductal gray matter (bregma — 6.04 to — 6.8 mm) and locus coeruleus (bregma — 9.68 to — 10.3 mm) were cut with a cryostat (Cryostat 1750, Leitz). Free-floating sections were washed four times with phosphate buffer containing 0.4% Triton X-100 and blocked with 2% donkey serum, 2% bovine serum antigen (Sigma, St. Louis MO, USA) for 60 min. Sections were incubated with goat antiserum (1:50 dilution) raised against C-18 of β-arrestin 2 (Catalog No. SC-6383, Santa Cruz, Santa Cruz, CA, USA) and rabbit antiserum (1:150 dilution) raised against amino acids 384–398 of the cloned rat μ-opioid receptor (No. Catalog PC165L Calbiochem Merck, Darmstadt, Germany). The 384–398 epitope corresponds to the complete exon 4 and the adjacent three amino acids of exon 3 of MOR-1 C terminal (Abbadie et al., 2000).

Once sections were washed three times in phosphate buffer with Triton, they were incubated for two additional hours at 37 °C with the secondary antibodies: tetramethyl rhodamine isothiocyanate-labeled donkey anti-rabbit, and fluorescein isothiocyanate-labeled donkey anti-goat (both obtained from Jackson Immunoresearch) diluted at 1:150. After three more washes, sections were mounted on glass slides
with antifade kit (Invitrogen, Carlsbad, CA, USA) to minimize photo-bleaching, and cover-slipped. Negative controls, designed to assess non-specific antibody binding, were conducted in parallel. In these controls, sections were treated as described but they were not exposed to primary antibody. Preliminary experiments were also conducted with different dilutions of primary and secondary antibodies, in an effort to optimize visualization of the two proteins of interest and minimize the background. Slides were kept in the dark until analysis.

Images of brain slices (0.45 μm optical thickness) were acquired with a Zeiss META 510 laser scanner microscope. Fluorescein isothiocyanate signal (green) was obtained after excitation with an argon (488 nm) laser using an emission window of 505–530 nm. Tetramethyl rhodamine isothiocyanate signal (red) was excited with a helium-neon laser (543 nm), and emission was detected through a 560 nm long-pass filter in multi-track mode to eliminate cross talk between the channels. The pinhole was 1 airy unit. Images were obtained with a 40× oil-immersion objective (numerical aperture: 1.3). Detector gain and background were adjusted to provide optimal fluorescent range; all images were acquired in the same conditions. The images were stored and analyzed with Zeiss laser scanner microscope 4.0 spl software. For analysis of whole cell immunoreactivity to μ-opioid receptor and to β-arrestin 2, the mean for total specific pixels on the surface of 30 neurons (10 per animal), was obtained with Zeiss 4.0 software and the data were utilized for the graphs shown in Figs. 4 and 5. For μ-opioid receptor internalization data shown in Fig. 6, the intensity of μ-opioid receptor and β-arrestin 2 signal was analyzed along of a line of 10 μm that started on the plasma membrane of each cell and crossed through the intracellular space. Specific signal distribution was obtained from at least five cells in each treatment and a representative cell with its distribution pattern is shown. All cell images were processed with Photoshop CS3 (Adobe Systems) using identical values for contrast and brightness in all cases.

2.7. Data and statistical analysis

Antinociception was evaluated by an increase in the latency to tail withdrawal as a function of time. The latencies of the tail-flick responses were converted to the percentage of maximum possible effect (%MPE) for each animal, at each time, according to the following formula: %MPE = [(test latency – baseline latency)/(cut-off time – baseline latency)] × 100%. The cumulative antinociceptive effect during the whole observation period was determined as the area under the curve for the %MPE (0–120 min) and these values were used for dose–response curves (Yoshikawa et al., 2007). Results are expressed as the mean ± S.E.M. for six animals per group. Comparisons between two experimental groups were done by Student’s t test, while comparisons among several drug treatments were done by a one-way analysis of variance followed by Dunnett’s test. In order to compare the time-course of the nociceptive effect produced by morphine alone or in combination with fentanyl, we used a two-way analysis of variance for repeated measures (factors: time and treatment) followed by Tukey test. The ED50 (i.e., the dose that caused 50% of maximum antinociception) and associated 95% confidence intervals were generated from standard non-linear regression analysis of the log dose–response curves (Prism 4.0, Graphpad Software, San Diego, CA, USA).

Morphine and fentanyl interaction was evaluated by isobolographic analysis (Tallarida, 2001) using dose–response curves in which the effects were expressed as the area under the curve of the time course for antinociception (Déciga-Campos et al., 2003; Granados-Soto and Argüelles, 2005; Yoshikawa et al., 2007). This approach was followed because both maximal responses and duration of analgesic effects were important for this study. The ED50 values of morphine and fentanyl were placed on the x- and y-axes, respectively, and the two points were connected with a line representing the theoretical additive effect of both drugs. According to this analysis, when the experimentally determined data points and their confidence interval lie along this line, the drug effects are additive (no interaction); when the points lie below this line, they are superadditive (synergy), and when they lie above this line, they are antagonistic. We administered fixed-ratio combinations of opioids (i.c.v.) in 1:3, 1:1 and 3:1 ratios of their respective ED50 fractions and the theoretical additive ED50 value was then compared with the experimental ED50 of the combination to determine whether there was a statistically significant difference. The degree of synergism was calculated by the isobolar relation (Tallarida, 2001, 2002): γ = a/ A + b/ B, where A and B are the doses of drug A (fentanyl alone) and B (morphine alone), respectively, that give the specified effect and (a,b) are the combination doses that produce the same effect. The quantities in the equation were obtained from the dose–response curves of drugs A, B, and their combinations. If γ = 1, the interaction was additive; if γ < 1, it was super-additive (synergy); and if γ > 1, it was sub-additive (antagonism). The program used for Statistics was SigmaStat (version 2.03, Jandel Scientific).

3. Results

3.1. Dose–response curves

Fig. 1 shows the time course of antinociception for increasing i.c.v. doses of morphine and fentanyl, and their corresponding dose–response curves. With few exceptions, peak effects were achieved 15 min after administration of both drugs. In general, high doses of morphine produced peak antinociceptive effects that lasted up to 30 min and declined rapidly thereafter, while fentanyl achieved peak levels at approximately 15 min and they gradually decreased towards control levels. As expected, fentanyl was similarly effective, but more potent than morphine. Thus, morphine produced a maximal antinociceptive effect of 7887 ± 353 area units and fentanyl of 6878 ± 739 area units. The calculated ED50 was 1.5 μg (CL0.95 0.9–2.6) for morphine and 0.034 μg (CL0.95 0.02–0.05) for fentanyl.

Once the type of interaction between fentanyl and morphine was determined, we selected an effective morphine dose (1 μg) to be used in combination with a low dose of fentanyl (1 ng) for internalization studies (Section 3.3). This drug proportion was chosen based on reports from the literature showing that a low dose of fentanyl or methadone (another β-arrestin biased ligand), increased morphine antinociceptive effects (Hashimoto et al., 2006; He and Whistler, 2005).

3.2. Isobologram

The results of the isobolographic analysis are depicted in Fig. 2. The oblique line between the x- and y-axes is the additive line, and the points in the middle are the theoretical additive points calculated from the 1:3, 1:1 and 3:1 combination of separate ED50 values. Table 1 shows the additive and experimental ED50 values and γ values. These data indicate a synergistic interaction between morphine and fentanyl because the experimental points lie far below the additive line and the interaction index values are < 1 for the three examined fixed-ratio combinations.

3.3. Combined actions of an effective morphine dose and a low dose of fentanyl

3.3.1. Nociception

Once the type of pharmacological interaction between these analgesics was determined, we studied whether the combination of an effective dose of morphine with a low, almost sub-effective fentanyl dose could also yield synergistic effects. The results are shown in Fig. 3. Fentanyl (1 ng) produced mild antinociception during the first 15 min after administration and no effect thereafter; this pattern
was similar in animals that received one or five fentanyl administrations (%MPE: 16.2 ± 4.4% and 14.6 ± 4.8%, respectively; n.s., Student’s t-test). A single morphine dose (1 μg) produced a 65.1 ± 11% increase in the latency to tail-flick that peaked at 15 min, remained stable for 30 min and decreased afterwards. The fifth morphine administration was not effective i.e., at this time, animals were already tolerant.

The combination of 1 μg morphine plus 1 ng fentanyl produced a peak effect of 89.1 ± 3% at 45 min and this effect lasted longer than the one produced by morphine alone in acutely- and chronically-treated animals. Although the efficacy of this morphine–fentanyl combination diminished by the fifth administration (peak effect: 72.3 ± 9.8% at 30 min), it was still significantly higher when compared with control or morphine-treated rats. Morphine–fentanyl coadministration was particularly effective after one hour of drug administration, when morphine was no longer effective on its own (panels A and C). Differences between the overall effect of antinociceptive treatments were also evident when the effects were expressed as area under the curve values (panels B and D).

3.3.2. Naloxone-precipitated withdrawal

Naloxone (31 μg) did not elicit abstinence signs in animals injected five times with a low dose of fentanyl (1 ng), morphine (1 μg) or morphine plus fentanyl (data not shown).

3.3.3. Mu opioid receptor internalization and β-arrestin 2 activity

In order to correlate antinociceptive effects with changes in μ-opioid receptor localization and density and β-arrestin 2 activity in periaqueductal gray and locus coeruleus, immunofluorescence studies were performed in animals treated once or five times with morphine and fentanyl, alone or in combination. Fig. 4 (upper panels) shows representative images from neurons stained with antibodies that recognize μ opioid receptors (red) and β-arrestin 2 in its active conformation (green) in animals that received one or five doses of each treatment. Active β-arrestin 2 immunoreactivity was barely detectable in control animals and in those that received one morphine dose. A significant increase in β-arrestin 2 activation was seen with the morphine–fentanyl combination in comparison with the other groups both in acutely- and chronically-treated animals (Fig. 4, lower panels). No significant changes in total μ-opioid receptor immunoreactivity were observed under any circumstances. Mu opioid receptors were located on the plasma membrane in cells of animals that received one injection of saline solution or morphine, while their distribution was more diffuse when morphine and fentanyl were coadministered (panel A and B, second column). This combination was associated not only with an

<table>
<thead>
<tr>
<th>Fentanyl–morphine combinations</th>
<th>Experimental ED50 ± S.E.M. (μg, i.c.v.)</th>
<th>Theoretical additive ED50 ± S.E.M. (μg, i.c.v.)</th>
<th>γ (interaction index)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:3</td>
<td>0.33 ± 0.04*</td>
<td>1.13 ± 0.28</td>
<td>0.29 ± 0.08</td>
</tr>
<tr>
<td>1:1</td>
<td>0.21 ± 0.05*</td>
<td>0.77 ± 0.18</td>
<td>0.28 ± 0.09</td>
</tr>
<tr>
<td>3:1</td>
<td>0.09 ± 0.02*</td>
<td>0.4 ± 0.05</td>
<td>0.23 ± 0.07</td>
</tr>
</tbody>
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* P<0.05 vs. the respective additive group; Student’s t test.
increase in β-arrestin 2 immunoreactivity, but also with μ-opioid receptor and β-arrestin 2 colocalization (yellow).

Fig. 5 shows the results obtained in locus coeruleus. The same pattern of changes observed in the periaqueductal gray was found in this brain area; i.e., there was an increase in β-arrestin 2 activity and μ-opioid receptor internalization with the morphine–fentanyl combination in acutely- and chronically-treated animals.

Fig. 6 analyzes the effect of different analgesic treatments on μ-opioid receptor and β-arrestin 2 signal redistribution in periaqueductal gray neurons. The quantitative fluorescence intensity vs. distance profiles was scanned along the straight arrow indicated in confocal images. Control group showed fluorescence principally in the plasma membrane. This pattern was also observed in animals treated with morphine or fentanyl alone. No differences in μ-opioid receptor subcellular localization were observed between acute and repeatedly treated animals. When the morphine-fentanyl combination was administered, fluorescence was not only found in the plasma membrane, but also in the cytoplasm, indicating μ-opioid receptor endocytosis. Similar results were seen in locus coeruleus (data not shown).

4. Discussion

Our results show that the combined treatment of morphine with fentanyl results in long-lasting synergistic antinociceptive effects after single or repeated administration, and that these actions are positively correlated with an increase in μ-opioid receptor endocytosis and β-arrestin 2 activity in the periaqueductal gray matter and locus coeruleus.

No differences in the time to reach peak effects and duration of antinociception produced by each separate drug were found in the present study probably due to the use of the i.c.v. route which precludes several pharmacokinetic processes. Using this administration route, morphine and fentanyl showed similar efficacy, but different antinociceptive potency. These results reproduce what has been seen in clinical practice and preclinical experiments with other administration routes where fentanyl has proven to be consistently, and significantly (up to a hundred times) more potent than morphine (Jeal and Benfield, 1997; Meert and Vermeirsch, 2005; Romero et al., 2010; Trescot et al., 2008).

The antinociception produced by morphine–fentanyl combination has been analyzed before, but only after a single injection and under different experimental conditions. Romero and coworkers found additive effects in the hot plate test when effective doses of morphine and fentanyl were coadministered to mice (Romero et al., 2010). Their study differs from ours in the use of different animal species, the response under study – paw lick is a supra-spinal response (Langerman et al., 1995) and tail-flick is a spinal reflex –, the administration route (s.c. vs. i.c.v.), and the drug proportions used in combination (effective vs. sub-effective doses of fentanyl). Also, Romero's study measured peak responses, whereas we analyzed cumulative effects during the whole observation period (2 h), which allowed antinociception evaluation at late times at which synergism became more evident (Fig. 3).

In another study, Bolan and coworkers reported only additive actions in the tail-flick test in mice treated with a combination of s.c. morphine plus fentanyl. They reached this conclusion using a quantal approach to assess antinociception (i.e., the percentage of animals that responded to a fixed thermal stimulus) and effective
doses of both compounds in combination (Bolan et al., 2002). Variations among results are not surprising because it has been reported that analgesic combination effects can change with the ratio of the two components of the drug pair, the level of effect, the site of injection, and the nociception test (Le Bars et al., 2001; Romero et al., 2010; Tallarida, 2001). Nonetheless, our study shows that synergistic effects between morphine and fentanyl can be observed within a wide dose range providing that long-lasting nociception is being evaluated.

Another significant finding was that coadministration of morphine and fentanyl produced analgesia under circumstances where morphine was no longer effective. Moreover, in spite of the enhanced antinociception observed in chronically treated animals we did not observe significant withdrawal signs after a high naloxone challenge in any of the groups studied. This suggests that no significant physical dependence occurred under our experimental conditions; i.e., the synergism found with morphine and fentanyl did not include this adverse effect, at least after five administrations. It is worth noting that although physical dependence is frequently intertwined with tolerance, each can occur independently (Aceto, 1990; Smith and Picker, 1998). In our laboratory we have found tolerance, but not dependence to the repeated administration of a morphine–dipyrone combination (Hernández-Delgadillo et al., 2003). With the combination used in the present study we cannot discount the possibility that dependence would occur with more prolonged treatments, but even in that case, the delay in physical dependence development would constitute a potential advantage of using this combination for pain treatment.

It has been considered that coadministration of two μ-agonists is an empirical approach without sound theoretical ground because if drugs act similarly, one could expect to see, at best, additive effects (Barrera et al., 2005). Nonetheless, the analgesic synergy found in the present study between morphine and fentanyl could be due to a mixture of mechanisms with subtle, but physiologically relevant differences.

Mu opioid agonists differ in their capacity to induce receptor signaling and trafficking. In particular, morphine has been described as

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**Fig. 4.** Coadministration of morphine and fentanyl (M + Fen) increases μ-opioid receptor (red) and β-arrestin 2 (green) colocalization (yellow) in periaqueductal gray. Rats received one (panel A) or five (panel B) i.c.v. administrations of saline (Control), 1 μg morphine (M), 1 ng fentanyl (Fen) or the combination of M + Fen. A representative neuron obtained from animals with each treatment is shown in the upper panels. Lower graphs show the pixels of specific fluorescence on the surface of at least 30 neurons per treatment. Data are presented as the mean±S.E.M. *P<0.05, **P<0.001 vs. control; Dunnett’s test.
a G-protein biased ligand, whereas fentanyl seems to preferentially act in a β-arrestin 2 dependent manner (Rajagopal et al., 2010). Both morphine and fentanyl produce receptor desensitization (Virk and Williams, 2008), but morphine provokes considerably less receptor internalization than fentanyl (Finn and Whistler, 2001; He et al., 2002; Johnson et al., 2005; Koch et al., 2005) and both ligands differ in receptor desensitization. Several authors have shown that morphine's ability to induce μ-opioid receptor internalization can be improved when morphine is combined with another opioid agonist with high internalization capacity. Hashimoto and colleagues studied the antinociception produced by acute i.t. administration of 2.5 μg morphine and 0.5 μg fentanyl alone or in combination, and correlated this effect with μ-opioid receptor internalization in the spinal dorsal horn neurons of rats. At the doses tested, morphine and fentanyl on their own were not effective in the hot plate test, but together they produced significant antinociception. The authors suggested that this was due to an increase in μ-opioid receptor endocytosis (Hashimoto et al., 2006). Our results agree with these data because we also found increased antinociception and enhanced μ-opioid receptor internalization with acute coadministration of morphine and fentanyl; moreover, present results extend these findings to two supraspinal areas (periaqueductal gray and locus coeruleus) that are central in pain regulation (Millan, 2002), tolerance and withdrawal (Scavone and Van Bockstaele, 2009; Williams et al., 2001). The fact that receptor endocytosis is positively correlated with increased antinociception in different anatomical sites suggests that the mechanisms regulating μ-opioid receptor endocytosis are similar at different levels of nociceptive pathways.

Fig. 5. Effects of acute and chronic coadministration of morphine and fentanyl (M+Fen) on μ-opioid receptor immunoreactivity (red), β-arrestin 2 activity (green) and μ-opioid receptor and β-arrestin 2 colocalization (yellow) in locus coeruleus. Rats received one (panel A) or five (panel B) i.c.v. administrations of saline (Control), 1 μg morphine (M), 1 ng fentanyl (Fen) or the combination of M+Fen. A representative neuron obtained from animals with each treatment is shown in the upper panels. Lower graphs show the pixels of specific fluorescence on the surface of at least 30 neurons per treatment. Data are presented as the mean±S.E.M. *P<0.05, **P<0.001 vs. control; Dunnett’s test.
This suggests that more than one receptor isoform could be involved in endocytosis of morphine-activated receptors because they formed dimers/oligomers. Because fentanyl, like methadone, is a biased \( \beta \)-arrestin ligand (Rajagopal et al., 2010), it is reasonable to suppose that fentanyl-activated receptors could promote endocytosis of morphine-stimulated receptors even after repeated administration. The fact that \( \beta \)-arrestin 2 activation increased with repeated administration of our analgesic combination supports this idea and suggests that \( \beta \)-arrestin 2 dependent signaling pathways remain active for a long time without showing substantial desensitization. Our results also agree with other authors who have found that \( \mu \)-opioid receptor endocytosis prevents tolerance development in simplified cell culture model systems (Koch et al., 2005) and other experimental preparations (reviewed in von Zastrow, 2010).

As previously mentioned, morphine and fentanyl differ not only in signaling preferences, but also in efficacy (Sirohi et al., 2008; Smith, 2008) and affinity for splice variants of the \( \mu \)-opioid receptor gene (Pasternak, 2004). Our results can be discussed in the light of different recent findings in the field. The antibody used in this work recognizes \( \mu \)-opioid receptor isoforms with the common C terminal sequence codified by exon 4 of the gene. With the exception of mMOR-1, those variants also have the N terminal amino acid sequence codified by exon 11 (Pan et al., 2009; Xu et al., 2011). Interestingly, in exon 11 knockout mice, fentanyl loses its antinociceptive efficacy to a much higher extent than morphine (Pan et al., 2009). This suggests that more than one receptor isoform could be involved in the morphine–fentanyl combination effects observed in our study.

In conclusion, the analgesic combination of morphine plus fentanyl (M+Fen) increases \( \mu \)-opioid receptor endocytosis in periaqueductal gray sections. Animals were subjected to one or five i.c.v. administrations of saline (C), 1 \( \mu \)g morphine (M), 1 ng fentanyl (Fen) or the combination of M+Fen at the same doses. Intensity of the \( \mu \)-opioid receptor and \( \beta \)-arrestin 2 signal was analyzed along of a line of 10 \( \mu \)m starting at the plasma membrane of each cell and crossing through the intracellular space. Distribution of specific red (\( \mu \)-opioid receptor) or green (\( \beta \)-arrestin 2) signal was obtained from the depicted cell and placed in the lower part of each photograph. Images show amplifications of a representative cell analyzed in each treatment.

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